A Section 508–conformant HTML version of this article is available at https://doi.org/10.1289/EHP13233.

Application of the Key Characteristics Framework to Identify Potential Breast Carcinogens Using Publicly Available *in Vivo*, *in Vitro*, and *in Silico* Data

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Background: Chemicals that induce mammary tumors in rodents or activate estrogen or progesterone signaling are likely to increase breast cancer (BC) risk. Identifying chemicals with these activities can prompt steps to protect human health.

Objectives: We compiled data on rodent tumors, endocrine activity, and genotoxicity to assess the key characteristics (KCs) of rodent mammary carcinogens (MCs), and to identify other chemicals that exhibit these effects and may therefore increase BC risk.

Methods: Using authoritative databases, including International Agency for Research on Cancer (IARC) Monographs and the US Environmental Protection's (EPA) ToxCast, we selected chemicals that induce mammary tumors in rodents, stimulate estradiol or progesterone synthesis, or activate the estrogen receptor (ER) *in vitro*. We classified these chemicals by their genotoxicity and strength of endocrine activity and calculated the overrepresentation (enrichment) of these KCs among MCs. Finally, we evaluated whether these KCs predict whether a chemical is likely to induce mammary tumors.

Results: We identified 279 MCs and an additional 642 chemicals that stimulate estrogen or progesterone signaling. MCs were significantly enriched for steroidogenicity, ER agonism, and genotoxicity, supporting the use of these KCs to predict whether a chemical is likely to induce rodent mammary tumors and, by inference, increase BC risk. More MCs were steroidogens than ER agonists, and many increased both estradiol and progesterone. Enrichment among MCs was greater for strong endocrine activity vs. weak or inactive, with a significant trend.

Discussion: We identified hundreds of compounds that have biological activities that could increase BC risk and demonstrated that these activities are enriched among MCs. We argue that many of these should not be considered low hazard without investigating their ability to affect the breast, and chemicals with the strongest evidence can be targeted for exposure reduction. We describe ways to strengthen hazard identification, including improved assessments for mammary effects, developing assays for more KCs, and more comprehensive chemical testing. https://doi.org/10.1289/EHP13233

Introduction

Breast cancer (BC) recently surpassed lung cancer to become both the most commonly diagnosed cancer type and leading cause of cancer death among women worldwide.[1] In the United States, it is the most commonly diagnosed invasive cancer[2,3] and the second leading cause of cancer death among women,[3] and the average lifetime risk for a woman to develop BC is 12.8% (more than double that of lung cancer, the second most common).[2,3] Moreover, BC especially affects younger women: Death rates from BC for women 20–49 years of age are more than double those for any other type of cancer among men or women,[4] and from 2010 to 2019, the rate of BC diagnoses among women <40 years of age rose 1.1% per year.[2] Identifying exposures that raise the risk of BC through established mechanisms, such as genotoxicity[5] and endocrine disruption,[6,7] can inform prevention and reduce the burden of disease.

Induction of mammary tumors in rodents is one useful proxy for identifying chemicals that increase BC risk in humans given that many of the target tissue structures (e.g., terminal ductal units) and pathways that lead to mammary tumors (hormonal activity, genotoxicity) are conserved between species.[8] Therefore, in 2007, we used databases from the International Agency for Research on Cancer (IARC), US National Toxicology Program (NTP), and others to identify 216 agents as potential breast carcinogens because they induce mammary tumors *in vivo* (i.e., they are mammary carcinogens; MCs).[9] This MC list has helped to prioritize chemicals for additional research,[10–17] identify data gaps and pitfalls in evaluating possible MCs,[18–24] inform studies of environmental exposures,[14,25–32] and target chemicals for exposure reduction.[25,33]

Since then, efforts to modernize chemical hazard identification have suggested a broader approach that incorporates mechanistic information about chemical bioactivity into carcinogen classifications, providing context for and reducing dependence on *in vivo* bioassays.[20,34–37] In recent years, IARC working groups developed a list of key characteristics (KCs) of carcinogens to identify common biological effects of known human carcinogens, providing a framework to identify other potential carcinogens based on having similar biological activities.[36,38,39] The KCs-of-carcinogens approach parallels that of the Hallmarks of Cancer,[40,41] except that whereas Hallmarks describe features of cancer cells and tissue, KCs describe effects of carcinogenic exposures,[42] such as genotoxicity, altered cellular signaling, increased cell proliferation, immunosuppression, inflammation, and epigenetic modifications.[35,36] Rarely does any single carcinogen exhibit all 10 KCs, but, in general, carcinogens act by one or more KC.[38] By focusing on mechanistic features, the KCs approach supports systematic and efficient identification of potential carcinogens that can then be assessed with more targeted studies.

Most established carcinogens act through mutagenic mechanisms,[39] represented by the two KCs of genotoxicity and alteration of DNA repair/genomic instability.[36] However, the other KCs point to additional pathways by which chemicals can promote tumors.[36,42] The close relationship between BC and hormone signaling[5,9,43–45] indicates that "receptor-mediated effects" is an especially relevant KC for BC. Indeed, BC is so closely tied to endocrine signaling that tumors are classified according to hormone receptor activity, particularly the estrogen receptor (ER) and the progesterone receptor (PR), and treatments to reduce BC risk and

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Supplemental Material is available online (https://doi.org/10.1289/EHP13233).
The authors declare no competing financial interests.
Received 27 April 2023; Revised 27 November 2023; Accepted 4 December 2023; Published 10 January 2024.

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recurrence block estrogen action.[46,47] Activation of the ER or PR increases cellular proliferation (a KC unto itself), and this is a critical mechanism by which endocrine-disrupting compounds (EDCs) promote mammary tumors.[48–51] As such, chemicals that increase estrogen or progesterone biosynthesis or activate estrogen or progesterone receptors are anticipated to promote mammary tumor development.[52]

In the present study, we aimed to identify and characterize BC-relevant exposures by updating the 2007 list of MCs, compiling information on their genotoxicity and endocrine activity, and extending the list to include chemicals that activate BC-relevant endocrine signaling pathways. We also calculated the enrichment of MCs for those biological effects (i.e., the proportion of MCs that exert these effects compared with all chemicals screened) and tested how well existing data on genotoxicity and two types of endocrine activity could predict whether a chemical is likely to induce mammary tumors in rodents. By investigating the overlaps between KCs and known MCs, we can better understand how KC data could predict BC hazards. Our objective was to integrate carcinogenicity and mechanistic bioactivity data to construct a more complete list of BC-relevant compounds and, in so doing, advance BC prevention by informing the design of BC studies and improving chemical testing and hazard identification. This list can also serve as a case study for applying the KCs framework to integrate *in vivo* and mechanistic data to identify chemicals that are likely to increase risk of an adverse outcome (in this case, BC).

Methods

To develop a list of BC-relevant chemicals, we integrated the types of evidence summarized in Figure 1. We gathered chemical identifiers from the US Environmental Protection Agency (EPA) 2021r1 database of DSSTox Identifiers Mapped to CAS Numbers and Names.[53] We compiled lists and calculated statistics in R (version 4.1.0; R Development Core Team). Code and input files are available on Github at https://github.com/SilentSpringInstitute/Kay-et-al-EHP-2024.

Chemicals That Induce Mammary Tumors in Rodents

We consulted the following authoritative sources to identify exposures that induced rodent mammary tumors (i.e., MCs): IARC Monographs (volumes 1–131),[54] NTP Technical Reports (nos. 1–602),[55] NTP *15th Report on Carcinogens* (*15th RoC*),[56] US EPA Integrated Risk Information System (IRIS),[57] US EPA Office of Pesticide Programs (OPP),[58,59] US EPA Toxicity Reference Database (ToxRefDB, version 2.0),[60,61] US EPA Toxicity Values Database (ToxValDB, version 9),[62] Lhasa Carcinogenicity Database[63] (LCDB, a continuation of the now-retired Carcinogenic Potency Database[64]), and the now-retired National Cancer Institute's (NCI) Chemical Carcinogenesis Research Information System (CCRIS).[65] We used our original list of rodent MCs from Rudel et al.[9] as a guide for identifying potential MCs and then classified them as MCs if they met the criteria described below.

We searched IARC Monographs[54] for the term "mammary" and included chemicals that significantly increased mammary tumors in at least one study by pairwise comparison at any dose. We used the NTP Chemical Effects in Biological Systems Organ Sites with Neoplasia[55] search tool for NTP technical reports and included chemicals where the NTP concluded that there was "positive," "clear," or "some" evidence for induction of mammary tumors. We searched the *15th RoC* pdf files for the term "mammary," reviewed summary conclusions, and listed chemicals with studies showing significant induction of mammary tumors.[56] We searched "mammary" in the US EPA IRIS website (https://iris.epa.gov/AdvancedSearch/) and included chemicals with mammary tumors listed as a critical effect. We searched the US EPA's ToxRefDB[60,61] and ToxValDB[62] and included chemicals with rodent cancer bioassays showing treatment-related increases in mammary tumors at any dose. We searched summary data in the LCDB,[63] which comprises all previously documented entries of the

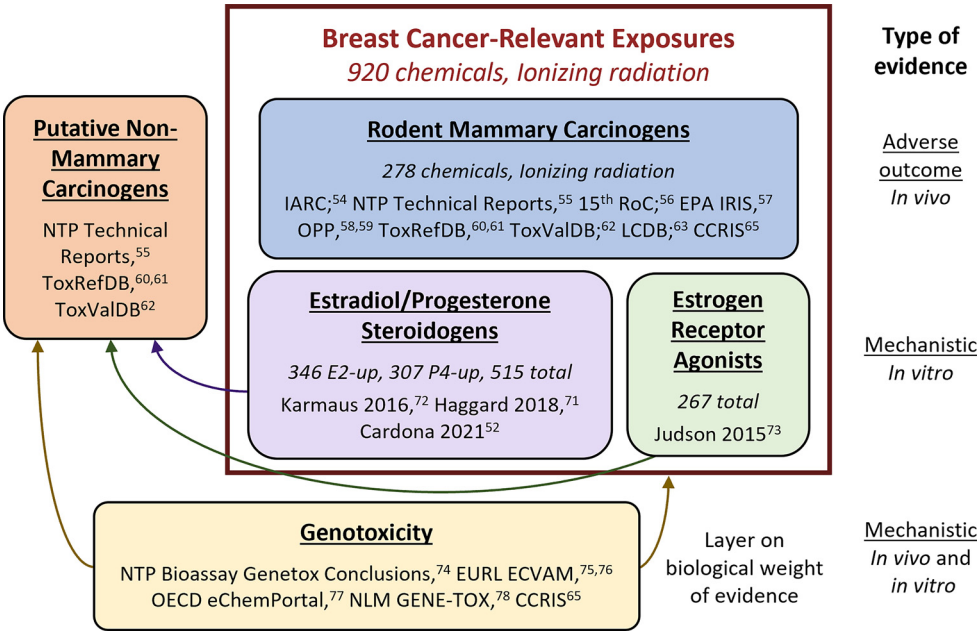

Figure 1. Information used to classify breast cancer-relevant chemicals. Note: 15th RoC, *15th Report on Carcinogens*; CCRIS, Chemical Carcinogenesis Research Information System; E2, estradiol; EPA, Environmental Protection Agency; EURL ECVAM, European Union Reference Laboratory for Alternatives to Animal Testing; GENE-TOX, Genetic Toxicology Data Bank; IRIS, Integrated Risk Information System; LCDB, Lhasa Carcinogenicity Database; NLM, National Library of Medicine; OECD, Organisation for Economic Co-operation and Development; OPP, Office of Pesticide Programs; P4, progesterone; ToxRefDB, Toxicity Reference Database; ToxValDB, Toxicity Values Database.

now-discontinued Carcinogenic Potency Database, as well as subsequent cancer assays,[66] for chemicals with "positive" evidence for tumors in the mammary gland, mammary tissue, and mammary ducts. We downloaded the archived NCI CCRIS database[65] and included chemicals with "positive" evidence for mammary tumors. We also included pesticides identified as having induced mammary tumors based on the US EPA OPP Registration Eligibility Decision (RED) and risk assessment documents as described in Cardona and Rudel 2020.[58]

We have previously described pitfalls and inconsistencies in cancer studies that can lead to unwarranted dismissal of mammary tumors.[9,58] Although we did not have the resources to review every cancer bioassay to determine whether mammary tumors were inappropriately dismissed, we reviewed studies for chemicals that we had previously flagged,[9,58] as well as chemicals for which mammary tumor induction was indicated as "equivocal" in NTP technical report conclusions or the *15th RoC*. For NTP technical reports, the *15th RoC*, and US EPA pesticide evaluations, we reviewed the underlying data and rationale and, if the chemicals were not already included as MCs based on listing by another source, we judged whether to classify them as MCs based on statistical significance, mechanistic evidence, and known pitfalls in evaluation of mammary tumors. We explain our rationale for these decisions in the "Discussion" section; in the Supplemental Material in "Supplemental discussion on dismissed or equivocal rodent mammary carcinogens"; and in Excel Table S2, and we noted the conclusion of the original source(s) in the MammaryTumorRefs column of Excel Tables S1 and S3–S5. CCRIS and LCDB do not explain their rationale for "equivocal" conclusions, so we noted their hit calls in Excel Tables S1–S5 but did not discuss them.

Some chemicals were listed as salts or parent compounds of salts, and it was not always clear which form was tested in the bioassay as summarized by the citing source. Thus, some entries may appear to be duplicates [e.g., 4-biphenylamine (listed by IARC,[54] *15th RoC*,[56] CCRIS[65]) and its hydrochloride (listed by LCDB[63])]. We listed chemicals exactly as they were listed in the source databases, and if the specific chemical tested was unclear, we listed the parent compound.

Putative Non-MCs

To evaluate whether chemicals with BC-relevant KCs are likely to be MCs, we developed a list of putative non-MCs—chemicals tested in a rodent cancer bioassay and not reported to induce mammary tumors. We identified chemicals tested in a cancer bioassay from three databases: NTP carcinogenicity technical reports from the NTP Integrated Chemical Environment (ICE),[67] and chemicals with rodent carcinogenicity studies recorded in US EPA's ToxRefDB[60,61] and ToxValDB.[62] Notably, ToxRefDB is the only resource that specifies tissues assessed in the bioassays, even if tumors were not observed (i.e., it lists negative results), so we could be certain that these bioassays included mammary assessment. ICE and ToxValDB do not specify which tissues were assessed, but the US EPA, NTP, and the Organisation for Economic Co-operation and Development (OECD) bioassay protocols require assessment of the mammary gland from at least the control and high-dose groups,[68–70] so we assumed that the mammary gland was assessed but could not confirm. In total, ICE, ToxRefDB, and ToxValDB listed bioassays for 977 chemicals, 127 of which we had listed as MCs. We classified the remaining 850 chemicals as putative non-MCs (Excel Table S5). IARC, *15th RoC*, US EPA IRIS, CCRIS, and LCDB were not useful to identify putative non-MCs because they do not systematically report bioassay results or verify that mammary tissue was assessed, and they include experimental studies that typically do not look at all tissues.

Chemicals That Increase Estradiol and Progesterone Steroidogenesis

To identify chemicals that stimulate synthesis of 17-β-estradiol (E2) and (P4), we relied on published data from the high throughput (HT) H295R assay.[52,71,72] In this assay, cultured human adrenocortical carcinoma (H295R) cells were stimulated with forskolin for 48 h and then exposed to the test chemical for 48 h, and the production of 11 hormones was measured in culture media.[71,72] Initially, 1,998 chemicals from ToxCast phases I, II, and III were tested in a single dose.[72] Subsequently, 656 chemicals were tested in a six-point concentration–response (CR) format; most were selected because they changed levels of at least 3 hormones by 1.5-fold or more in the single-dose test.[71,72] Two chemicals in CR testing were highly cytotoxic, and one chemical had data quality flags, so these were excluded from analyses,[71] leaving 653 chemicals with CR data. Detailed methods for the H295R assay are available in Haggard et al.[71] and Karmaus et al.[72]

For our list of chemicals that increased E2 or P4 synthesis, we excluded the hormones and hormone substrates E2, 17-α-estradiol, 17-α-ethinylestradiol, equilin, estriol, estrone, progesterone, 17-α-hydroxyprogesterone, 17-methyltestosterone, 4-androstene-3,17-dione, 5-α-dihydrotestosterone, androsterone, dehydroepiandrosterone, and testosterone propionate because hormones and their substrates are measured in the assay, so treatment with such chemicals may confound measurements of *de novo* steroid production. Given that we excluded these chemicals, the results are indicated as not applicable (NA) in Excel Tables S1 and S3–S5, and they are not included as part of the analyses summarized in Tables 1 and 2. After filtering out hormones and substrates, there were 1,984 chemicals tested in single-dose and 639 tested in CR assays.

Hits for the single-dose assay were determined from positive hit calls listed in Karmaus et al. 2016 supplementary Table 4 for estradiol_up and prog_up (hitc = 1).[72] Chemicals run multiple times in single dose were assigned hit calls based on whether they tested positive or negative more often (e.g., clorophene increased E2 synthesis one out of six times and is therefore indicated as negative). Chemicals that induced E2 or P4 production in the CR assay[71] were classified by efficacy and potency into borderline-, low-, medium-, and high-effect categories, as described in Cardona and Rudel.[52] Briefly, Cardona and Rudel classified E2-up and P4-up chemicals as positive if they *a*) increased synthesis by $\geq$1.5-fold over controls at any concentration, *b*) significantly increased synthesis at a concentration $\leq$33 μM, and *c*) had an adjusted maximal mean Mahalanobis distance >0. These chemicals were then ranked by these criteria, and the top 25% were assigned a "high" effect score, the middle 50% "medium," and the bottom 25% "lower."[52] Note that borderline chemicals were those with a positive hit call in the initial analysis[71] but that did not increase synthesis by $\geq$1.5-fold or only significantly increased synthesis at the highest concentration tested; some of these may be false positives, and the activities of some chemicals with high-dose effects may have been underestimated.[52] The remaining chemicals screened in this study did not meet the authors' criteria for a positive hit call for E2 or P4,[71] so these are indicated as nonsignificant (ns) in our tables.

We summarized H295R test results by merging the lists of E2 and P4 steroidogens from single-dose and CR testing. Because CR testing is more robust and less prone to false positives or false negatives than the single-dose assay, chemicals that increased E2 or P4 synthesis in single-dose but not in CR testing were classified as negative. Chemicals that increased E2 or P4 in single-dose testing and that were not tested in CR assays, or that were borderline-active in CR testing, are indicated in summary tables with an asterisk because the strength of evidence for these chemicals to increase steroidogenesis is lower.

ER Agonists

We identified ER-active chemicals from the supplemental data file S2 published by Judson et al.[73] In that study, 45 reference chemicals were tested in 18 *in vitro* ToxCast assays that measure ER-regulated pathways, including receptor binding and cellular proliferation, and those data were normalized to 17-α-ethinylestradiol and integrated to produce area-under-the-curve (AUC) scores for ER agonism and antagonism.[73] This testing and modeling approach was applied to a library of 1,812 chemicals with CR data from the 18 ToxCast assays for ER activity, and the authors used a threshold of AUC $\geq$0.1 to define chemicals with clear agonist/antagonist activity. Here, we classified chemicals with an AUC $\geq$0.7 as having high activity, 0.7 > AUC $\geq$0.4 as medium activity, and 0.4 > AUC $\geq$0.1 as low activity (Excel Tables S1 and S3–S5). Because Judson et al. indicated that an AUC <0.1 could reflect interferences in assay results,[73] we applied a second threshold of 0.1 > AUC $\geq$0.01 for borderline ER agonism or antagonism. Some chemicals were borderline agonistic and antagonistic, so we designated these as having mixed borderline activity. We considered chemicals with agonist and antagonist AUCs <0.01 to be inactive.

Genotoxic Chemicals

We ascertained chemical genotoxicity from results compiled in six databases from international and US agencies. From the NTP Bioassay Genetox Conclusion Dataset,[74] we extracted data from Ames mutagenicity, *in vivo* and *in vitro* micronucleus, and *in vivo* comet assays. From the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) Genotoxicity and Carcinogenicity Consolidated Databases of Ames positive and negative chemicals,[75,76] we collected results of mutagenicity (bacterial and mammalian), micronucleus (*in vitro* and *in vivo*), chromosomal aberration (*in vitro* and *in vivo*), and *in vivo* unscheduled DNA synthesis assays. From the OECD eChemPortal,[77] we collected data from *in vitro* and *in vivo* mutation, transformation, micronucleus, chromosomal aberration, unscheduled DNA synthesis, sister chromatid exchange, comet, and DNA adduct assays classified as reliable with or without restrictions. From the National Library of Medicine (NLM) Genetic Toxicology Data Bank (GENE-TOX),[78] we compiled data from *in vitro* and *in vivo* micronucleus, chromosomal aberration, mutation, mitotic recombination, unscheduled DNA synthesis, and sister chromatid exchange assays. From CCRIS,[65] we compiled *in vitro* genotoxicity data, including mutation (bacterial and mammalian), unscheduled DNA synthesis, micronucleus, and chromosomal aberration assays. In total, these databases included 17,894 chemicals with genotoxicity data. We classified chemicals as genotoxic if they had at least one positive result in any assay, nongenotoxic if all valid assays were negative, and inconclusive if no tests returned interpretable results (Excel Tables S1 and S3–S5).

KCs of Mammary Developmental Toxicants

We compared our list of BC-relevant chemicals (MCs, E2/P4 steroidogens, and ER agonists; Excel Table S1) to those of the 30 mammary gland developmental toxicants identified by Rudel et al.,[44] compiling data for steroidogenesis, ER agonism, and genotoxicity of the developmental toxicants with the same methods as above (Excel Table S4). This 2011 review emerged from the 2009 Mammary Gland Evaluation and Risk Assessment Workshop in Oakland, California, a convening of >65 scientists, public health advocates, and risk assessors, including experts in breast biology. The authors compiled the list of 30 mammary gland developmental toxicants through an "extensive PubMed literature review and examination of the citations," although they acknowledge that "a few relevant studies may be missing,"[44] and the list does not include studies published after 2011.

KCs of MCs: Calculating Enrichment and Predictivity

To determine whether MCs are more likely to have E2/P4 steroidogenic, ER-agonistic, or genotoxic effects than other chemicals—i.e., enrichment—we compared the fraction of MCs that tested positive for these activities against *a*) the fraction of all chemicals that tested positive in those assays and *b*) the fraction of putative non-MCs that tested positive using Fisher exact test [fisher.test() in the R Stats package]. We also calculated the ability of these mechanistic activities to predict if a chemical was an MC. To do this, we compared the results from steroidogenesis, ER agonism, and genotoxicity assays for MCs vs. putative non-MCs using standard calculations of specificity, sensitivity, and balanced accuracy:

$$Sensitivity\,(\%) = \frac{TP}{TP+FN} \times 100,$$

$$Specificity\,(\%) = \frac{TN}{TN+FP} \times 100,$$

$$Balanced\,Accuracy\,(\%) = \frac{Sensitivity + Specificity}{2} \times 100,$$

where *TP* (true positive) represents MCs that test positive for the effect; *FN* represents MCs that test negative for the effect; *TN* represents non-MCs that test negative for the effect; and *FP* represents non-MCs that test positive for the effect. Finally, we calculated trends for increasing strength of endocrine activities among MCs and putative non-MCs with two-sided Cochran–Armitage test [CochranArmitageTest() in the R DescTools package].

Results

We applied and evaluated a KC approach to identify likely breast carcinogens, focusing on receptor-mediated effects (KC 8[36]), specifically estrogenic and progestogenic signaling, given that these hormones are particularly relevant to breast carcinogenesis.[48–51] We defined BC-relevant chemicals as those that have been shown to induce mammary tumors in rodents (i.e., mammary carcinogens; MCs) and those that activated estrogenic or progestogenic signaling in either of two *in vitro* screens. For these BC-relevant chemicals, we also gathered data on genotoxicity (KC 2[36]), given that this is another important pathway to BC.[5] We assessed whether the KCs of estrogenic and progestogenic action and genotoxicity could predict the adverse outcome of mammary tumors by comparing the enrichment of these activities among MCs vs. chemicals that did not induce mammary tumors in a cancer bioassay.

BC-Relevant Chemicals

Overall, we identified 921 BC-relevant exposures, including 278 chemicals and ionizing radiation that induced mammary tumors in rodents, as well as 642 additional chemicals that had E2/P4 steroidogenic[52,71,72] (515 chemicals) or ER agonistic[73] (267 chemicals) activity *in vitro* (Figure 1 and Excel Table S1). Four hundred twenty-one BC-relevant chemicals were genotoxic,[65,74–78] and 485 exposures had more than one BC-relevant effect.

MCs. The updated search expanded our previous list of 216 MCs[9] to 279 exposures that induced mammary tumors *in vivo* based on studies that we gathered from the IARC, NTP, US EPA, and other authoritative databases (Figure 1 and Excel Table S1; chemicals that induce mammary tumors denoted as "MC" in the "MammaryTumorEvidence" column, and citations for mammary tumor induction listed in the "MammaryTumorRefs" column).

Table 1. E2/P4 steroidogenesis, ER agonism, and genotoxicity of chemicals tested in the assays and enrichment of these activities among MCs.

Effect	Chemicals tested (n)	Chemicals positive [n (%)]	MCs tested (n)[54–63,65]	MCs positive [n (%)]	MCs not tested (n)	p-Value[a]
E2 up (single dose)[72]	1,972	290 (15)	72	18 (25)	201	0.027*
P4 up (single dose)[72]	1,496	197 (13)	65	16 (25)	208	0.015*
E2 or P4 up (single dose)[72]	1,982	422 (21)	72	24 (33)	201	0.020*
E2 and P4 up (single dose)[72]	1,486	65 (4)	65	10 (15)	208	8.1×10^{-4}*
E2 up (CR)[71]	639	266 (42)	39	23 (59)	234	0.044*
P4 up (CR)[71]		275 (43)		22 (56)		0.13
E2 or P4 up (CR)[71]		404 (63)		28 (72)		0.31
E2 and P4 up (CR)[71]		137 (21)		17 (44)		0.0027*
E2 up (total)[b]	2,003	346 (17)	73	23 (32)	200	0.0044*
P4 up (total)[b]		307 (15)		23 (32)		8.3×10^{-4}*
E2 or P4 up (total)[b]		515 (26)		29 (40)		0.0098*
E2 and P4 up (total)[b]		138 (7)		17 (23)		1.3×10^{-5}*
ER agonist[73]	1,812	92 (5)	75	11 (15)	203	0.0019*
ER borderline agonist[73]		149 (8)		8 (11)		0.40
ER mixed borderline[73]		26 (1)		3 (4)		0.11
ER agonistic (any)[73]		267 (15)		22 (29)		0.0015*
ER antagonist[73]		18 (1)		0 (0)		1
ER borderline antagonist[73]		79 (4)		2 (3)		0.77
ER antagonist (any)[73]		123 (7)		5 (7)		1
Endocrine disrupting (any)	2,279	684 (30)	82	42 (51)	196	8.3×10^{-5}*
EDC+		369 (16)		31 (38)		3.6×10^{-6}*
Genotoxic[65,74–78]	17,894	7,582 (42)	227	209 (92)	51	2.2×10^{-16}*
Endocrine disrupting and genotoxic	1,456	246 (17)	76	35 (46)	202	1.1×10^{-8}*
EDC+ and genotoxic		140 (10)		27 (36)		3.8×10^{-9}*

Note: MCs are chemicals that induce mammary tumors in rodents; E2/P4 (total) integrates single dose[72] and CR[71]; ER agonistic (any) represents the sum of agonist, borderline agonist, and mixed borderline; ER antagonistic (any) represents the sum of antagonist, borderline antagonist, and mixed borderline; EDCs involve the integration of steroidogenesis and ER agonism. CR, concentration–response (format); E2, estradiol; EDC, endocrine-disrupting compound; ER, estrogen receptor; MC, mammary carcinogen; P4, progesterone. *Statistically significant ($p<0.05$).

[a]Fisher exact test comparing proportion of MCs positive vs. proportion of all chemicals positive.

[b]Chemicals tested in single dose only counted as positive if they were not tested or also positive in CR.

Notable additions to the list included several halogenated solvents, drinking water disinfection byproducts, benzidine-based dyes, and >30 pesticide ingredients. For 28 chemicals we classified as MCs (including 11 new additions to the original MC list), one or more references described mammary tumor induction as equivocal or dismissed it, although 22 of these had at least one other reference indicating that the mammary tumors were treatment-related (Excel Tables S1 and S2). Half of these chemicals are active pesticide ingredients for which the US EPA OPP was the entity that dismissed or questioned the tumors, including for the widely used malathion, atrazine, and triclopyr. The rationales for the US EPA dismissing or questioning carcinogenicity were mostly related to the following: inconsistent decisions about considering fibroadenomas as tumors, reductions in body weight at high doses that reduced mammary tumors and confounded dose–response trends, dismissal of nonmonotonic dose responses, assertion of a lack of mechanistic relevance to humans, and flawed study design, interpretation, and statistical comparisons (described in more detail in the "Discussion" section; in the Supplemental Material in "Supplemental discussion on dismissed or equivocal rodent mammary carcinogens"; and in Excel Table S2).

We updated several entries from our previous list. Specifically, we removed *N*-nitrosodibutylamine and wood dust methanol extract because subsequent reviews from the *15th RoC*[56] and IARC,[79] respectively, concluded that mammary tumors were not induced by these chemicals. We removed magnetic radiation because, although it promoted the development of chemically initiated mammary tumors, tumor induction by magnetic radiation alone was not shown.[80] We generalized the listings of other radiation sources (e.g., X-rays, neutrons, tritium) to "ionizing radiation."[81] Finally, we replaced the entries in our 2007 report for "bracken fern extracts" and "conjugated estrogens" with the specific chemicals that induced mammary tumors (respectively, ptaquiloside and *p*-ecdysone; and estradiol valerate, estradiol dipropionate, and estrone benzoate).

Endocrine disruptors. The KC "receptor-mediated effects" is highly relevant for chemicals associated with BC, especially for receptors involved in E2 and P4 signaling.[48–51] Using US EPA HT *in vitro* testing data, we identified chemicals that activate the ER or increase E2 or P4 synthesis (Figure 1) and classified them as BC-relevant based on strong evidence that these hormonal activities increase BC risk.[52,82–84] Our complete BC-relevant chemicals list combines the rodent MCs with the ER agonists and E2/P4 steroidogens identified through US EPA *in vitro* screening (Figure 1).

We identified ER agonists using data published by Judson et al.,[73] who computationally integrated results from 18 *in vitro* ToxCast assays that measure ER-regulated pathways to predict whether a chemical is an ER agonist or antagonist. Their integration of ToxCast CR data yielded AUC values for each chemicals' relative magnitudes of agonist and antagonist activities at the ER, normalized to 17-α-ethinylestradiol ($AUC_{agonist\text{-}ethinylestradiol}=1$). Of the 1,812 chemicals with CR data from these 18 assays, they classified 92 (5%) as ER agonists ($AUC_{agonist}\geq0.1$),[73] which we further stratified as 10 with high ($AUC_{agonist}\geq0.7$), 13 with medium ($0.7>AUC_{agonist}\geq0.4$), and 69 with low ($0.4>AUC_{agonist}\geq0.1$) agonistic activity. Judson et al. set a cutoff of AUC ≥0.1 for "clear" agonist/antagonist activity, but several of their weak-agonist reference chemicals fell below this cutoff.[73] We therefore created an additional category of borderline-active chemicals with $0.1>AUC\geq0.01$, classifying 175 (10%) chemicals as borderline agonists or as having mixed borderline activity (both $0.1>AUC_{agonist}\geq0.01$ and $0.1>AUC_{antagonist}\geq0.01$) (Excel Table S1; Judson et al. values shown in the AUC.Agonist and AUC.Antagonist columns, and our classifications in the ERactivity column). Of the 75 chemicals we classified as rodent MCs that were

Table 2. E2/P4 steroidogenesis, ER agonism, and genotoxicity of chemicals tested for carcinogenicity, enrichment of these activities among MCs, and predictivity.

Effect	Non-MCs tested (n)[60,62,67]	Non-MCs positive [n (%)]	MCs tested (n)[54–63,65]	MCs positive [n (%)]	p-Value[a]	Sensitivity (%)	Specificity (%)	Balanced accuracy (%)
E2 up (single dose)[72]	437	73 (17)	72	18 (25)	0.098	25	83	54
P4 up (single dose)[72]	409	60 (15)	65	16 (25)	0.067	25	85	55
E2 or P4 up (single dose)[72]	447	113 (25)	72	24 (33)	0.15	33	75	54
E2 and P4 up (single dose)[72]	399	20 (5)	65	10 (15)	0.0044*	15	95	55
E2 up (CR)[71]	202	83 (41)	39	23 (59)	0.052	59	59	59
P4 up (CR)[71]		88 (44)		22 (56)	0.16	56	56	56
E2 or P4 up (CR)[71]		126 (62)		28 (72)	0.28	72	38	55
E2 and P4 up (CR)[71]		45 (22)		17 (44)	0.0086*	44	78	61
E2 up (total)[b]	451	97 (21)	73	23 (32)	0.071	32	78	55
P4 up (total)[b]		95 (21)		23 (32)	0.051	32	79	55
E2 or P4 up (total)[b]		146 (32)		29 (40)	0.23	40	68	54
E2 and P4 up (total)[b]		46 (10)		17 (23)	0.0031*	23	90	57
ER agonist[73]	460	17 (4)	75	11 (15)	6.0×10^{-4}*	15	96	55
ER borderline agonist[73]		39 (8)		8 (11)	0.51	11	92	51
ER mixed borderline[73]		4 (1)		3 (4)	0.061	4	99	52
ER agonistic (any)[73]		60 (13)		22 (29)	8.0×10^{-4}*	29	87	58
ER antagonist[73]		3 (1)		0 (0)	1	0	99	50
ER borderline antagonist[73]		12 (3)		2 (3)	1	3	97	50
ER antagonist (any)[73]		19 (4)		5 (7)	0.36	7	96	51
Endocrine disrupting (any)	485	183 (38)	82	42 (51)	0.028*	51	62	57
EDC+		114 (24)		31 (38)	0.0089*	38	76	57
Genotoxic[65,74–78]	657	492 (75)	227	209 (92)	4.4×10^{-9}*	92	25	59
Endocrine disrupting and genotoxic	349	96 (28)	76	35 (46)	0.0024*	46	72	59
EDC+ and genotoxic		56 (16)		27 (36)	3.4×10^{-4}*	36	84	60

Note: MCs are chemicals that induce mammary tumors in rodents; non-MCs are chemicals that were tested in a rodent cancer bioassay and were not reported to induce mammary tumors; E2/P4 (total) integrates single dose[72] and CR[71]; ER agonistic (any) represents the sum of agonist, borderline agonist, and mixed borderline; ER antagonistic (any) represents the sum of antagonist, borderline antagonist, and mixed borderline; EDCs involve the integration of steroidogenesis and ER agonism. CR, concentration–response (format); E2, estradiol; ER, estrogen receptor; MC, mammary carcinogen; P4, progesterone. *Statistically significant ($p<0.05$).
[a]Fisher exact test comparing proportion of MCs positive vs. proportion of putative non-MCs positive.
[b]Chemicals tested in single dose only counted as positive if they were not tested or also positive in CR.

included in ER activity modeling by Judson et al.,[73] 11 (15%) met the criteria for ER agonists, and another 11 met our criteria for borderline agonists (including mixed borderline), for a total of 22 (29%) ER-agonistic MCs (Table 1). No MCs met the criteria for being ER antagonists ($AUC_{antagonist} \geq 0.1$), which is consistent with the hypothesis that ER activation in the breast increases BC risk, as well as with the clinical use of ER antagonists to suppress breast carcinogenesis (e.g., tamoxifen, raloxifene[85–87]). We classified two MCs as borderline antagonists (3-iodo-2-propynyl-*N*-butylcarbamate and C.I. Acid Red 114) and three MCs as having mixed borderline ER activity (1,4-benzenediamine, 17-[(1-oxohexyl)oxy)pregn-4-ene-3,20-dione [hydroxyprogesterone caproate], and 4,4′-methylenebis(*o*-toluidine)].

We further identified 515 chemicals that stimulated E2 or P4 biosynthesis in the H295R *in vitro* assay (excluding hormones and substrates, see the "Methods" section and also Excel Table S1, columns E2up_onedose through HormoneSummary, for results).[52,71,72] In the single high-dose assay, 422/1,982 chemicals (21%) induced E2 or P4 synthesis, with 290/1,972 (15%) increasing E2, 197/1,496 (13%) increasing P4, and 65/1,486 (4%) increasing both (Table 1 and Excel Table S1, E2up_onedose and P4up_onedose columns for Karmaus et al. hit calls).[72] Of the MCs that were tested at a single dose, 18/72 (25%) increased E2 and 16/65 (25%) increased P4; 24/72 (33%) increased E2 or P4, and 10/65 (15%) increased both. Of the 639 chemicals we considered from the H295R assay performed in CR (excluding hormones and substrates), 266 (42%) increased synthesis of E2, 275 (43%) increased P4, 404 (63%) increased either, and 137 (21%) increased both (Table 1 and Excel Table S1, E2up_CR and P4up_CR columns for Cardona and Rudel hit calls).[52,71] Of the 39 MCs included in the CR study, 23 (59%) increased E2, 22 (56%) increased P4, 28 (72%) increased either, and 17 (44%) increased both (Table 1).

We summarized results of E2 and P4 induction in H295R by combining results from both single-dose and CR assays (HormoneSummary column in Excel Tables S1 and S3–S5). Given that the CR assay format is more robust and less prone to false positives, we classified chemicals that increased E2 or P4 synthesis only in the single-dose format but not in CR as negative, and we excluded these from our list of BC-relevant chemicals unless they were also an MC or ER agonist based on the criteria described in the "Methods" section, "Chemicals that Induce Mammary Tumors in Rodents" and "Estrogen Receptor Agonists," respectively. Chemicals that were tested only in a single dose and increased E2 or P4 synthesis and chemicals with borderline activity in CR are marked with an asterisk in the HormoneSummary column of Excel Tables S1 and S3–S5 to indicate weaker evidence of effects. In total, 2,003 chemicals were tested for steroidogenicity,[52,71,72] and after applying the criteria above, we considered 515 (26%) to increase E2 or P4, including 296 categorized as active and 219 categorized as borderline active (see the "Methods" section, "Chemicals that Increase Estradiol and Progesterone Steroidogenesis"). Seventy-three of the chemicals tested for steroidogenesis were in our list of rodent MCs, of which we considered 23 (32%) to increase E2, 23 (32%) to increase P4, 29 (40%) to increase either, and 17 (23%) to increase both (Table 1).

In Excel Tables S1 and S3–S5, we have indicated the evidence for endocrine-disrupting activity in the EDC column: ER agonists ($AUC \geq 0.1$) and chemicals that increased E2 or P4 steroidogenesis with low, medium, or high activity in CR are designated EDC+, reflecting the higher confidence for endocrine-related effects of

these chemicals; chemicals that increased only E2/P4 steroidogenesis in the single dose, were borderline-steroidogenic in CR, or weakly activated the ER ($0.1 > AUC_{agonist} \geq 0.01$, with or without borderline antagonism) are designated EDC ~, indicating lower confidence; and chemicals that were not E2/P4 steroidogens or ER agonists are designated EDC–. It is important to note that, although ER antagonists are by definition endocrine disruptors, for this analysis we are defining EDCs to refer only to chemicals with evidence for *increasing* estrogenic or progestogenic signaling through steroidogenesis or ER agonism. Notably, 10 chemicals that were negative for steroidogenesis or ER agonism were not tested in the other assay, and we were only able to assess two types of BC-relevant endocrine activity with reliable HT screens (in the "Discussion" section in "*In vitro* and mechanistic data"). Thus, some EDC- chemicals may in fact be EDCs. We also indicated P4 as EDC+ because, although we excluded it from our H295R analyses and it was a borderline ER agonist,[73] it is a key hormone of interest and exposure to P4 would activate a BC-relevant pathway.[51] Throughout the paper, the term EDC refers to EDC+ and EDC ~ chemicals unless otherwise specified.

For more a nuanced consideration of the strength (potency plus efficacy) of endocrine-disrupting effects, we also created a category for the top EDC score for each chemical tested in H295R-CR[52,71,72] or in the ER pathway model,[73] given that these assays provided a measure of effect size. In Excel Tables S1 and S3–S5, this column is populated with the classifier of the strongest endocrine effect for each chemical, so, for example, a chemical that was high E2-up, low P4-up, and borderline ER agonistic received a "high" top EDC score. Chemicals that were inactive in these assays received a top EDC score of "none." With this approach, 94 chemicals we classified as BC-relevant (16 of them MCs) met our criteria for having high activity in E2/P4 production in the H295R CR format or ER agonism in the ER activity model, 158 (12) as medium, 116 (2) as low, 221 (11) as borderline, 111 (36) with no significant activity, and 222 (203) that were not tested in these screens.

Significantly, because we could not identify a reliable screen for PR activity (see the "Discussion" section in "*In vitro* and mechanistic data"), the strength of some PR agonists' endocrine activities is underestimated. For example, we excluded P4 from our H295R-CR analysis, so its top EDC score is "borderline" based on ER agonism. Other PR agonist MCs whose strength of endocrine activity may be underestimated include 17-α-hydroxyprogesterone (top EDC score of low), norethindrone (medium), and lynestrenol (none).

Genotoxicity of BC-Relevant Chemicals

Having identified 921 BC-relevant MCs and EDCs, we classified those agents according to evidence for the KC of genotoxicity. Because genotoxicants can induce cancer-initiating mutations,[36,40] it was unsurprising that 209 (92%) of the 227 chemical MCs tested[65,74–78] had reported genotoxic activity (Table 1). Ionizing radiation was not included in databases of chemical testing, but its genotoxicity is well established;[81,88–92] given that radiation is not a chemical, it is not included in Table 1 but it is indicated as genotoxic in the list of BC-relevant exposures (Excel Table S1).

EDCs are more likely to increase BC risk if they also have the KC of genotoxicity because these activities can both initiate and promote carcinogenesis.[36,40] Of the 417 E2/P4 steroidogenic and ER-agonistic BC-relevant chemicals tested for genotoxicity,[65,74–78] 246 showed a positive result in at least one assay (Excel Table S1). Limiting to only higher-confidence EDCs, there were 140 genotoxic EDC+ chemicals, including 27 MCs (Excel Table S3). These MCs with endocrine-disrupting and genotoxic properties include several widely used pesticides (malathion, parathion, atrazine, simazine, and ametryn), endogenous and synthetic hormones (E2, estriol, estrone, 17-α-ethinylestradiol, P4, diethylstilbestrol, and mestranol), and dye components (C.I. Azoic diazo component 112 [benzidine], 3,3′-dimethylbenzidine and its dihydrochloride, C.I. Disperse Black 6 and its dihydrochloride, *o*-aminoazotoluene, 1,4-benzinediamine, and 5-nitro-*o*-anisidine). Furthermore, 3,3′-dimethylbenzidine and its dihydrochloride salt, C.I. Azoic diazo component 112, *o*-aminoazotoluene, isoeugenol, 1,4-benzenediamine, and diethylstilbestrol were considered genotoxic MCs with both steroidogenic and ER-activating properties (Excel Tables S1 and S3).

Mammary Gland Developmental Toxicants

Prenatal and early life exposure to EDCs can alter mammary gland development in humans and animals in ways that raise BC risk.[44,93–102] We therefore compared the endocrine-disrupting and genotoxic properties of the 30 chemicals we identified as rodent mammary gland development disruptors in 2011[44] (Excel Table S4). We classified 15 of these as BC-relevant (MC, E2/P4-steroidogenic, or ER agonistic), 3 as EDC-, and 13 were not included in US EPA's *in vitro* steroidogenesis[52,71,72] or ER activity[73] screens. Fourteen BC-relevant mammary developmental toxicants were E2/P4 steroidogenic or ER agonistic: the top EDC score was "high" for 4 of these chemicals, "medium" for 3, "low" for 4, and "borderline" for 3. In the H295R screen for steroidogenesis, 3 mammary developmental toxicants were shown to increase E2 synthesis, 3 increased P4, 1 increased both, and 9 were not active. Ten developmental toxicants were classified as ER agonists, 3 had borderline agonistic activities, and 2 (tamoxifen and fulvestrant) were medium-strength ER antagonists. Because these ER antagonists are used to both treat BC and prevent recurrence,[85,86,103] it is not surprising that they alter mammary gland development, and these observations reinforce the importance of ER agonism as a KC for breast carcinogens. Finally, 16 of the developmental toxicants showed evidence of genotoxicity,[65,74–78] 12 of which also had BC-relevant steroidogenic[52,71,72] or ER agonistic[73] activity. Of the 30 mammary developmental toxicants, 6 were in our list of MCs, and many of the others [benzyl butyl phthalate, dichlorodiphenyltrichloroethane (DDT), zearalenone, perfluorooctanoic acid (PFOA), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and polybrominated diphenyl ethers] have been found to affect the breast in humans.[44,82,83]

Putative Non-Mammary Carcinogens

Because a goal of this study was to determine whether E2/P4 steroidogenesis, ER agonism, and genotoxicity are indicators of whether a chemical is likely to increase BC risk, we identified putative non-MCs to compare the KC activities among chemicals that do and do not induce mammary tumors in rodents. We found 850 chemicals with bioassays listed in ICE,[67] ToxValDB,[62] and ToxRefDB[60] that we classified as putative non-MCs because they were not on the MC list (i.e., mammary tumors were not induced in the bioassay) (Excel Table S5). Of the 451 putative non-MCs with H295R data,[52,71,72] 97 increased E2, 95 increased P4, 146 increased either, and 46 increased both; of the 460 included in ER activity modeling,[73] 17 were agonists and 43 were borderline agonists; and of the 657 included in genotoxicity databases,[65,74–78] 492 showed at least one positive result (Table 2 and Excel Table S5). Ninety-six putative non-MCs met our criteria for being genotoxic EDCs (see the "Methods" section). Note that based on our reviews of mammary tumors in cancer bioassays, as described in in the "Discussion" section in "Two-year cancer bioassay" and in

Cardona and Rudel[58] and Kay et al.,[83] we expect that some of these putative non-MCs may in fact be rodent MCs.

Enrichment of MCs for KCs

To test our hypothesis that endocrine disruption and genotoxicity are important KCs of breast carcinogens, we calculated the prevalence of endocrine-disrupting and genotoxic activities among MCs compared with all chemicals tested, that is, enrichment, by the Fisher exact test (Table 1). With few exceptions, MCs were significantly enriched for increasing steroidogenesis, activating the ER, inducing genotoxicity, and having combinations of those effects. Of the 76 MCs tested for genotoxicity and endocrine activity (ER agonism or E2 or P4 steroidogenesis), 35 (46%) were positive for both, compared with 246 (17%) of the 1,456 chemicals tested that had data for both characteristics (Table 1). More than half of the MCs tested for steroidogenesis or ER agonism were active (42 EDCs of 82 MCs tested), and most of those endocrine-disrupting MCs were also genotoxic (35 genotoxic EDCs of 76 MCs tested). MCs were more than twice as likely to be higher-confidence endocrine disruptors (EDC+) compared with the group of all chemicals tested: 38% (31/82) of MCs were EDC+ compared with 16% (369/2,279) of all chemicals tested (2.3-fold higher, $p = 3.6 \times 10^{-6}$), and 36% (27/76) of MCs were genotoxic EDC+ compared with 10% (140/1,456) of all chemicals tested (3.7-fold higher, $p = 1.5 \times 10^{-8}$).

Interestingly, among endocrine-related effects, p-values for enrichment among MCs were lowest for increasing synthesis of both E2 and P4 rather than just one (6 E2-only, 6 P4-only, 17 E2+P4, of 73 MCs tested). This corresponds to >3-fold enrichment for MCs that increased both hormones (17/73 vs. 138/2,003 of all chemicals tested). It is also notable that greater proportions of MCs were found to be steroidogenic than ER-active, even though the historical emphasis has been on detecting activity at the ER to characterize chemicals' endocrine-disrupting and BC-promoting potential. These data suggest that steroidogenesis—rather than ER activation—may be a more prevalent mechanism by which chemicals stimulate mammary tumorigenesis.

We also compared the enrichment of genotoxic and endocrine activities among MCs vs. putative non-MCs (Table 2). The fractions of putative non-MCs active in endocrine-related assays were similar to those of the full set of chemicals tested in those assays, so results were similar whether MCs were compared against all chemicals tested (Table 1) or against putative non-MCs (Table 2), although statistical comparisons were stronger for the larger sample size of all chemicals tested. A greater proportion of non-MCs displayed genotoxicity[65,74–78] compared with all chemicals tested, so enrichment of MCs for genotoxicity (with or without endocrine activity) was less pronounced for comparisons against non-MCs, although it was still significant. Ultimately, MCs were significantly enriched for all three BC-relevant mechanistic effects whether compared with all chemicals tested (Table 1) or putative non-MCs (Table 2), bolstering confidence in these findings.

We refined our analysis of how activity in EDC assays relates to likelihood of inducing mammary tumors by comparing enrichment of top EDC scores (see the "Results" section, "Endocrine Disruptors") among MCs and putative non-MCs. First considering only endocrine effects, we found that MCs were 2.6 times more likely to have top EDC scores categorized as "high" ($p = 0.0015$) and 1.4 times less likely to have no top EDC score ($p = 0.0033$) compared with non-MCs (Table 3 and Figure 2). There was a statistically significant trend for stronger endocrine activities among MCs compared with putative non-MCs ($p = 2.1 \times 10^{-4}$).

Table 3. Enrichment of MCs for strength of endocrine activity compared with putative non-MCs.

Top EDC score	Non-MCs [n (%)][60,62,67]	MCs [n (%)][54–63,65]	Fold-difference	p-Value
High	38 (8.1)	16 (21)	2.6	0.0015*[a]
Medium	46 (9.8)	12 (16)	1.6	0.16[a]
Low	30 (6.4)	2 (2.6)	0.41	0.29[a]
Borderline	52 (11)	11 (14)	1.3	0.44[a]
None	306 (65)	36 (47)	0.72	0.0033*[a]
Total	472	77	—	—
Trend				2.1×10^{-4}*[b]

Note: —, not applicable; EDC, endocrine-disrupting compound; MC, mammary carcinogen. *Statistically significant ($p < 0.05$).
[a]Fisher exact test comparing MCs vs. non-MCs.
[b]Two-sided Cochran–Armitage trend test for strength of endocrine activity among MCs vs. non-MCs.

We also wanted to understand whether a chemical's likelihood of inducing mammary tumors could be predicted from a combined assessment of its genotoxicity and the strength of its endocrine activity. MCs were approximately three times more likely to be genotoxic and have "high" ($p = 0.0032$) or "medium" ($p = 0.0084$) top EDC scores compared with putative non-MCs, and again the trend for higher EDC scores among MCs vs. non-MCs was significant ($p = 0.0012$) (Table 4 and Figure 3). There were only five nongenotoxic MCs with EDC data, so power was limited for evaluating enrichment in this set. Nevertheless, we found that MCs were 14 times less likely to both be nongenotoxic and lack a top EDC score ($p = 2.6 \times 10^{-5}$) compared with non-MCs, and the trend for increasing EDC strength was still significant when comparing nongenotoxic MCs vs. non-MCs ($p = 0.0024$). Thus, although MCs had the lowest p-values for enrichment of genotoxicity overall (Tables 1 and 2), the combination of genotoxicity and endocrine potency was more informative than genotoxicity or endocrine activity alone (Tables 3 and 4). If the potential for a chemical to induce mammary tumors depended on genotoxicity and not endocrine effects, then the proportion of MCs vs. non-MCs that were genotoxic would be similar among all magnitudes of endocrine activity; instead, genotoxicants with stronger endocrine activity were more likely to be MCs, whereas genotoxicants without significant activity in H295R-CR[52,71] or the integrated ER model[73] had a similar likelihood of inducing mammary tumors or not (Table 4).

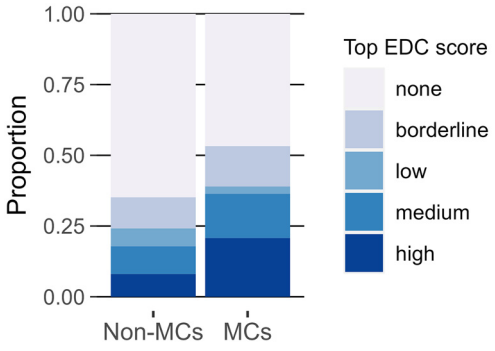

Figure 2. Proportions of top EDC scores among MCs and putative non-MCs (values in Table 3). Top EDC scores for each chemical are assigned based on the strongest effect in E2 or P4 steroidogenesis (H295R CR format[71]) or ER agonism,[73] with the criteria of high: top 25% of E2- or P4-inducers by Cardona and Rudel 2021 ranking[52] or ER Agonism AUC ≥0.7; medium: middle 50% of E2- or P4-inducers or 0.7> ER AUC ≥0.4; low: bottom 25% of E2- or P4-inducers or 0.4> ER AUC ≥0.1; borderline: statistically significant E2 or P4 induction not reaching Cardona and Rudel 2021 criteria or 0.1> ER AUC ≥0.01; and none: no statistically significant induction of E2 or P4 or ER AUC <0.01. Note: AUC, area under the curve; CR, concentration–response (format); E2, estradiol; EDC, endocrine-disrupting compound; ER, estrogen receptor; MC, mammary carcinogen; P4, progesterone.

Table 4. Enrichment of MCs for strength of endocrine activity and genotoxicity compared with putative non-MCs.

Top EDC score	Genotoxicity	Non-MCs [n (%)][60,62,67]	MCs (n)[54–63,65]	Fold-difference	p-Value
High	+	21 (6.3)	13 (18)	2.93	0.0032[a]
Medium	+	18 (5.4)	11 (15)	2.89	0.0084[a]
Low	+	17 (5.1)	2 (2.8)	0.56	0.55[a]
Borderline	+	30 (8.9)	8 (11)	1.26	0.51[a]
None	+	158 (47)	32 (45)	0.96	0.79[a]
Trend[b]	+				0.0012*[b]
High	−	3 (0.9)	2 (2.8)	3.15	0.21[a]
Medium	−	10 (3.0)	1 (1.4)	0.47	0.7[a]
Low	−	4 (1.2)	0 (0)	0.00	1[a]
Borderline	−	10 (3.0)	1 (1.4)	0.47	0.7[a]
None	−	65 (19)	1 (1.4)	0.070	2.6×10^{-5}
Trend[b]					0.0024*[b]
Total		336	71	NA	NA

Note: EDC, endocrine-disrupting compound; MC, mammary carcinogen. *Statistically significant ($p < 0.05$).
[a]Fisher exact test comparing MCs vs. non-MCs.
[b]Two-sided Cochran–Armitage trend test for strength of endocrine activity among MCs vs. non-MCs.

Predicting BC Hazard Based on KCs

Although induction of mammary tumors in rodents is an imperfect predictor of human breast carcinogenicity,[39] we used rodent mammary tumor induction as a proxy for potential human breast carcinogenesis because they are similarly influenced by genotoxicity and hormonal signaling.[8] We therefore calculated how well steroidogenesis, ER activity, and genotoxicity could predict a chemical's likelihood of being an MC. This information could direct the application of these assays to screen potential breast carcinogens. We compared the results from KC assays for MCs vs. putative non-MCs using standard calculations of sensitivity (ability to detect true positives), specificity (detecting true negatives), and balanced accuracy (integration of sensitivity and specificity).

Endocrine-related assays generally showed high specificity but low sensitivity (Table 2). The overall high specificity reflects the fact that putative non-MCs were unlikely to have endocrine activity. For example, only 4% (17/460) of non-MCs were ER agonists and only 10% (46/451) increased both E2 and P4, corresponding to specificities of 96% and 90%, respectively. Despite enrichment of MCs for endocrine activities, many MCs were not active in these assays, leading to low sensitivity. This is consistent with the expectation that there are many biological pathways to BC aside from ER agonism and E2/P4 steroidogenesis, including genotoxicity,[5] other types of endocrine signaling,[16,51,104] and multiple other KCs.[16,105,106] On the other hand, genotoxicity was highly sensitive for detecting MCs but poorly specific given that many non-MCs were also genotoxic.

Combining sensitivity and specificity, the balanced accuracy of any combination of endocrine or genotoxicity data fell between 50% and 61% (Table 2). The greatest balanced accuracy was achieved for chemicals increasing both E2 and P4 in the H295R CR assay (61%) and for genotoxic EDC+ chemicals (60%).

Discussion

This updated list of 921 BC-relevant exposures is, to our knowledge, the first to use a KC approach that combines 279 rodent MCs with 642 additional chemicals that have mechanistic evidence for biological activities likely to increase BC risk. Based on extensive evidence that estrogenic and progestogenic pathways promote breast carcinogenesis,[5,16,44,52] (reflecting KC 8, receptor-mediated effects[36]) we included chemicals that activate the ER or increase synthesis of E2 or P4 as BC-relevant along with the rodent MCs. Lack of high quality screening data for other BC-relevant mechanisms, such as PR activation, limited

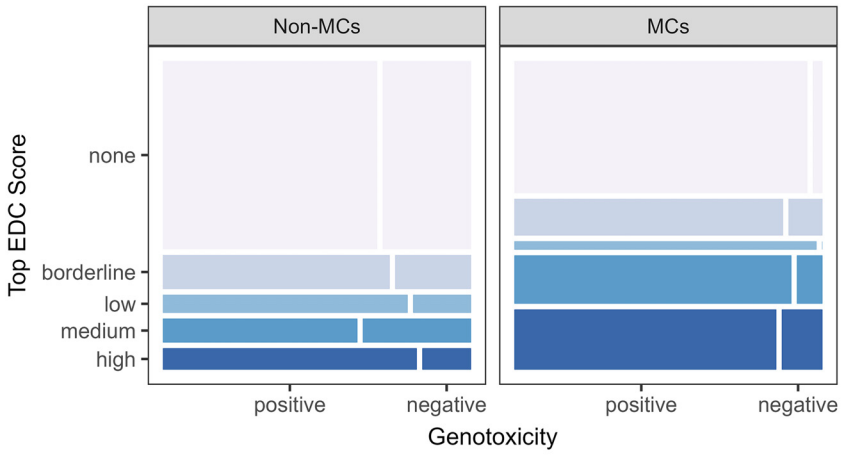

Figure 3. Mosaic plots of top EDC scores and genotoxicity among MCs and putative non-MCs (values in Table 4). Top EDC scores for each chemical are assigned based on the strongest effect in E2 or P4 steroidogenesis (H295R CR format[71]) or ER agonism,[73] with the criteria of high: top 25% of E2- or P4-inducers by Cardona and Rudel 2021 ranking[52] or ER Agonism AUC ≥0.7; medium: middle 50% of E2- or P4-inducers or 0.7> ER AUC ≥0.4; low: bottom 25% of E2- or P4-inducers or 0.4> ER AUC ≥0.1; borderline: statistically significant E2 or P4 induction not reaching Cardona and Rudel 2021 criteria or 0.1> ER AUC ≥0.01; and none: no statistically significant induction of E2 or P4 or ER AUC <0.01. Charts portray scores in order from "high" at the bottom to "none" at the top. Note: AUC, area under the curve; CR, concentration–response (format); E2, estradiol; EDC, endocrine-disrupting compound; ER, estrogen receptor; MC, mammary carcinogen; P4, progesterone.

our ability to include them. We also compiled genotoxicity data for these BC-relevant chemicals as further evidence suggesting the potential to increase BC risk, given that genotoxicity is a prevalent KC (KC 2[36]) of many carcinogens. Based on their activity in these KCs, the chemicals on our list—particularly the most potent ones—are more likely than most to increase BC risk, and we recommend prioritizing them for additional research and precautionary regulation.

KCs of MCs

As chemical testing paradigms shift toward more mechanistic approaches, this new application of the KCs of carcinogens provides insights into the etiology of BC and strategies for carcinogen identification. Our goals in compiling data on chemical genotoxicity and endocrine activity were 3-fold: *a*) to highlight two KCs of known MCs, *b*) to demonstrate that these KCs are enriched among MCs, and *c*) to identify other chemicals that exhibit these KCs and may therefore be MCs as well.

Genotoxicity is a KC of most known carcinogens,[7,79,81,107–110] and it has historically been the first consideration in predicting carcinogenic potential. Because genotoxicants can initiate and promote carcinogenesis,[36,40] it is not surprising that 92% of the 227 MCs included in the databases we used were genotoxic.

The KC "receptor-mediated effects" is also highly salient for BC, particularly for effects mediated through the ER and PR. More than 70% of BC cases are hormone responsive,[111–114] and numerous experimental and epidemiological studies have linked E2 and P4 disruption to BC, with increased hormonal activity correlating with increased risk of BC,[7,111,113,115,116] and inhibited E2 signaling correlating with reduced BC risk and severity.[7,117]

Notably, many MCs on our list demonstrated both genotoxicity and the ability to stimulate estrogenic or progestogenic signaling; of 76 MCs tested, 35 (46%) showed both effects, a significant enrichment compared with all chemicals tested (17%, 246/1,456; Table 1) and compared with putative non-MCs (28%, 96/349; Table 2). This combination of genotoxic and endocrine activity may explain why certain chemicals, such as the commonly used experimental carcinogen 7,12-dimethylbenz(a)anthracene (DMBA, a potent E2 steroidogen and genotoxicant), predominantly induce mammary tumors.[118] Of the 642 BC-relevant EDCs not previously identified as MCs, 211 (33%) were also genotoxic, but 115 of these genotoxic EDCs did not have cancer studies recorded in ICE,[67] ToxValDB,[62] or ToxRefDB.[60] We consider these genotoxic EDCs, which include several widely used pesticides and dye components, to be strong candidates for regulation based on their mechanistic activities and also priorities for *in vivo* or epidemiological investigation as possible breast carcinogens, especially the strongest EDCs.

When we evaluated the ability for H295R, ER activity, and genotoxicity assays to predict mammary carcinogenicity, we found that positive results in these assays were significantly enriched among MCs compared with non-MCs (Table 2). Furthermore, we found that MCs were significantly enriched for having high EDC scores compared with non-MCs, and they were significantly less likely to test negative for endocrine and genotoxic effects (Tables 3 and 4). These trends were confirmed with positive associations between MCs and strength of endocrine activity, regardless of genotoxicity. We found a high degree of specificity for most endocrine-related effects (i.e., most putative non-MCs tested negative, Table 2), reinforcing the importance of endocrine pathways in BC and indicating that activity in these selected assays can be used to flag chemicals as likely BC hazards. However, the low sensitivity of endocrine activity (i.e., many MCs tested negative) reinforces the notion that these assays miss other mechanisms of breast carcinogenesis. Genotoxicity, on the other hand, was a highly sensitive but weakly specific predictor of MCs, given that both MCs and putative non-MCs were likely to be genotoxic. Because balanced accuracy requires good sensitivity and specificity, better predictions require new knowledge about BC mechanisms and assays to test them (e.g., PR activity). In addition, a more quantitative characterization of assay results [e.g., half-maximal activities (AC_{50}s), genotoxic effect sizes] and integration of toxicokinetics/toxicodynamics could also improve their predictive power.

One limitation of our KC predictivity analysis is that, although we found MCs to be significantly enriched for endocrine and genotoxic activities, these measures depend on the set of chemicals tested. For example, although zero MCs were antagonists in the ER model, the proportion was not statistically significantly different from all chemicals tested or from putative non-MCs, perhaps because only 18 of the 1,812 chemicals tested were antagonists, limiting statistical power. In addition, most chemicals selected for H295R screening in CR were tested because they significantly altered multiple hormone levels in the single-dose screen. Thus, the enrichment of E2/P4 steroidogenesis for MCs in H295R CR was statistically weak (Tables 1 and 2) because the chemicals tested in CR had already been shown to affect steroidogenesis. The most meaningful comparison for H295R data was in the combination of single-dose and CR testing, given that these numbers reflected the full set of chemicals assessed for steroidogenesis, and we placed more weight on results from the more robust CR assay format (see the "Methods" section). Relatively few MCs were included in H295R and ER activity screens, so enrichment calculations would have been more statistically robust with a larger number of chemicals to compare. Fewer than 30% of chemical MCs were tested in these screens (82/278 chemical MCs tested in either H295R or the integrated ER model). In fact, 45 MCs had no data on genotoxicity or endocrine disruption from any of the sources we considered (Excel Table S1). Finally, some putative non-MCs may be false negatives (discussed below and by Kay et al.[83]), so enrichment calculations may be over- or underestimates.

Overall, the consistent and significant enrichment of MCs for genotoxicity and multiple measures of endocrine activity across two comparison groups (vs. all chemicals tested and vs. putative non-MCs) demonstrates the robustness of our findings. The significant enrichment (Tables 1 and 2) and trend for increasing strength of endocrine activity among MCs (Tables 3 and 4) supports the utility of H295R and ER activity assays to predict a chemical's likelihood of increasing BC risk. More extensive chemical screening for endocrine activity would strengthen statistical comparisons. Given the enrichment of MCs for endocrine and genotoxic effects, and the well-established association between BC risk and exposure to hormonally active and genotoxic agents,[5,82] many of the EDCs identified here can be plausibly anticipated to increase BC risk, particularly if they are also genotoxic or have strong activity in endocrine assays. Validation of the *in vitro* endocrine effects considered here with *in vivo* or human studies could test this hypothesis and clarify risks associated with these compounds.

Carcinogenesis and KCs Assays: Strengths and Limitations

Although the biological effects we used as a basis for creating this list of chemicals that may increase BC risk (mammary carcinogenicity, E2/P4 steroidogenesis, ER activity, and genotoxicity) are useful for this purpose, we also want to highlight limitations with the methods used to measure these activities and identify opportunities to strengthen them. These limitations are important

considerations both for interpretation of our results and for future application of the KCs approach to hazard identification.

Two-year cancer bioassay. The 2-y rodent bioassay has been heavily relied upon for carcinogenicity testing because it effectively predicts human cancer risk,[37,56] especially for genotoxic chemicals that induce cancer-initiating mutations.[36,40] The major strength of the cancer bioassay is that it is a controlled long-term laboratory study of chemical exposures *in vivo*, isolating the specific effects of a chemical in an animal that is similar to humans in metabolism and toxicokinetics/toxicodynamics.[37] *In vivo* studies like the cancer bioassay are essential to validate *in vitro* and *in silico* chemical testing methods.

However, several aspects of the 2-y bioassay design constrain its ability to identify breast carcinogens, particularly EDCs.[119,120] First, unlike genotoxicants, which are considered tumor-initiating,[37] many EDCs appear to influence carcinogenesis through tumor promotion or developmental alterations that sensitize tissues to hormonal stimuli.[43,121–123] The interplay between genotoxic, hormonal, and developmental processes reduces the ability of the standard cancer bioassay to identify MCs and complicates the interpretation of mammary tumors.[83]

Second, testing chemicals in isolation misses effects of coexposures, especially for tumor promoters—such as EDCs—that may require initiating events to induce cancer. This gap is important because people are continually exposed to mixtures of genotoxicants and EDCs. Additive effects of multiple environmentally relevant levels of EDCs can produce adverse outcomes that single exposures do not, and low numbers of initiated mutant cells can be promoted to tumorigenesis through endocrine disruption.[124–130] If bioassays tested combined exposures, it is likely more EDCs (including some putative non-MCs) would produce mammary tumors in test conditions.

Furthermore, in the 2-y bioassay, animals begin chronic exposure to test chemicals after they are weaned,[68–70,131] but it is well known that BC risk is influenced by exposure during a range of windows of susceptibility (WoS), including prenatal, perinatal, pubertal, parous, and menopausal periods.[82,132–136] Because some of these WoS occur before dosing begins in the assay,[120] a lack of mammary tumors in the bioassay does not demonstrate that the chemical would not produce tumors following early life exposure.[137]

Beyond the timing of exposures and collections in the cancer bioassay, methods for mammary tissue collection and evaluation also limit the ability to detect cancerous lesions.[83] First, US EPA and OECD guidelines require microscopic assessment of tissues from only the control and high-dose animals; evaluation of tissue from lower dose groups is only required when lesions are detected macroscopically or if effects are observed at the high dose.[68,70,83] This approach impedes identification of carcinogens that induce tumors at lower doses, as can occur in nonmonotonic dose responses or when high-dose toxicity masks the effects of lower doses (discussed below). In addition, histopathological assessments of the mammary gland in US EPA, NTP, and OECD bioassays are typically performed on transverse cross-sections, cut perpendicular to the skin, rather than longitudinal sections, cut parallel to the skin.[68–70,83] Transverse sections yield very little mammary tissue, making it unlikely that these samples would contain microscopic lesions.[83,138] Microscopic assessment of longitudinal sections and whole-mount mammary glands would vastly improve the assay's ability to detect neoplastic lesions arising from chemical exposures, particularly if all dose groups were assessed.[83]

Another issue with the 2-y cancer bioassay is that because it is cost-, labor-, and time-intensive, many potential carcinogens have not been tested. Identifying agents that exhibit KCs of carcinogens, such as genotoxicity and endocrine disruption, can help prioritize chemicals for bioassay testing and guide precautionary action. We identified 115 genotoxic EDCs that did not have a bioassay recorded by NTP, ToxValDB, or ToxRefDB (Excel Table S1), and we consider these priority candidates for testing in a cancer bioassay that uses relevant WoS and appropriate techniques as described above.

Another source of uncertainty in our list of MCs is inconsistent reporting of mammary tumor findings by study authors, sponsors and regulatory agencies.[9,52,58] We reviewed US EPA OPP carcinogenicity studies for 24 pesticides and NTP technical reports for 14 additional chemicals we had previously flagged for inconsistency or uncertainty in conclusions about mammary tumors,[9,58] identifying 28 chemicals that we classified as MCs and noting that our conclusions differed from some study authors or regulators. Most cases where mammary tumors were dismissed or questioned came from the US EPA OPP. Below, we describe five recurring themes that led to dismissal of mammary tumors. All 28 cases of MCs with dismissed or equivocal evidence for mammary tumors are summarized in Excel Table S2 and described in greater detail in the Supplemental Material in "Supplemental discussion on dismissed or equivocal rodent mammary carcinogens." A careful review of bioassays conducted on BC-relevant chemicals, keeping in mind the issues discussed in this section, could identify some MCs that have previously been inappropriately designated as non-MCs.

Theme 1: fibroadenomas. Although fibroadenomas (FBAs) occur as benign lesions in rats, they are clinically significant in humans and can be legitimately interpreted as tumors.[139–142] FBA growth in humans is likely hormonally mediated, signaling exposure to endocrine-active compounds, and some studies suggest they can progress to malignancy or increase the risk of developing other breast tumors.[139–146] Furthermore, human FBAs can be confused with carcinomas or cancer metastases; distinguishing them can necessitate invasive diagnostic methods, such as biopsy; and large FBAs may require surgery.[143,144] Opinions differ whether FBAs in rodents can progress to malignancy and whether they predict malignant tumorigenesis in humans[8,147]; however, hormonal stimuli and well-established MCs, including DMBA, induce and increase both FBAs and malignant tumors in rodents.[148–152] For all these reasons, we consider FBAs as significant abnormal sequelae of chemical exposures that reflect changes relevant to human breast carcinogenesis. In the Supplemental Material in "Supplemental discussion on dismissed or equivocal rodent mammary carcinogens," we describe examples where significant FBA induction was dismissed, or where FBA incidence was combined with mammary adenomas and carcinomas during analysis to eliminate the statistical significance of the latter tumor types.

Theme 2: nonmonotonic dose responses. Many toxicological assessments assume that chemicals exert their strongest effects at higher doses and are weaker at lower doses.[131,153] However, numerous studies have shown EDCs eliciting nonmonotonic dose responses, including in the mammary gland,[10,21,33,153–156] because hormones (and therefore disruptions in hormonal signaling) produce different effects at different concentrations. For example, the US EPA dismissed the significant induction of mammary tumors from the pesticides malathion[157] and alachlor[158] in the middle- and low-dose groups, respectively, because tumor incidence in the high-dose group was not statistically significantly different from controls (Excel Table S2 and Supplemental Material in "Supplemental discussion on dismissed or equivocal rodent mammary carcinogens").

Theme 3: high-dose toxicity. High doses of chemicals in the bioassay can render food unpalatable or cause systemic toxicity, either of which can reduce the animal's body weight. Because lower body weight reduces mammary tumor incidence,[9,159,160] it can mask what would be a treatment-related increase in tumors.

In some studies, mammary tumor induction in high-dose groups becomes significant if results are adjusted for body weight,[160] but this adjustment is inconsistently applied.[161–165] We found several examples where statistically significant increases in mammary tumors at lower doses were dismissed owing to the lack of further increases at higher doses (nonmonotonic dose–response), and many of these were accompanied by body weight reductions (Excel Table S2 and Supplemental Material in "Supplemental discussion on dismissed or equivocal rodent mammary carcinogens"). We expect that standardized approaches for maintaining assay sensitivity in the presence of altered body weight would improve 2-y bioassay accuracy and consistency.

Theme 4: mechanistic relevance to humans. When a pathogenic mechanism in a test animal is not present in humans, findings from the assay may not apply to human cancer risk. However, we found that for chlorotriazine herbicides (including atrazine, simazine, and propazine), the proposed mechanism for induction of mammary tumors in rodents was dismissed as not relevant to humans without adequate evidence. These herbicides consistently induced mammary tumors in female Sprague–Dawley rats,[166–168] and study sponsors and the US EPA OPP have proposed that the tumors result from an attenuated luteinizing hormone surge, causing persistent high levels of circulating E2 that stimulate mammary cell proliferation.[167,169,170] We question the assertion that this mechanism is not relevant in humans[166,169,170] owing to multiple evidentiary gaps and logical flaws in their conclusions (Supplemental Material in "Supplemental discussion on dismissed or equivocal rodent mammary carcinogens"). These include a lack of measurements of E2 levels and mammary cell proliferation, dismissal of genotoxicity, and conflation of rat strains with different sensitivities. Indeed, atrazine has been shown to activate aromatase and increase E2 synthesis in human[71,171–174] and rat[174] cells, providing a plausible mechanism for atrazine to promote mammary tumorigenesis in both species.

Theme 5: study design and comparator selection. Reviewers of cancer bioassays sometimes compare tumor rates in treated animals against those in concurrently dosed controls, and in some cases also compare with historical controls pooled from years of bioassays on the same strains of rodents.[69,131] The former approach is the default and higher-confidence approach, although historical controls can be useful for rare tumors.[69,131] Caution should be used when comparing with historical controls because rodent strains can undergo genetic drift, shifting the rate of spontaneous tumors over time, and differences in housing conditions and feed can affect spontaneous tumor development.[175,176] Several MCs in our list showed significant increases in mammary tumors compared with matched or in-house controls but were at the high end of the historical control range; others showed marginal increases compared with matched controls but exceeded the historical control range (Excel Table S2 and Supplemental Material in "Supplemental discussion on dismissed or equivocal rodent mammary carcinogens"). In addition, statistical tests are affected by the number of animals compared, so significance may be weakened by comparing too few matched controls or a large number of historical controls that have been affected by changes in genetics, housing, or food. These are important considerations in assessing tumor induction, particularly when assessments are subject to other pitfalls as described above (e.g., FBA, low body weight) or if reviewers do not present their rationale for dismissing tumors.

Extent and quality of databases for rodent mammary tumors. A challenge for identifying chemicals that do and do not induce tumors at any site is that systematic, comprehensive, well-maintained databases of cancer bioassays (and other experimental studies) are not readily available. In the present study, we used nine databases to identify MCs and three of those databases to identify putative non-MCs (see the "Methods" section). Two databases that we relied on to identify MCs (CCRIS[65] and Carcinogenic Potency Database[64]/LCDB[63]) are no longer maintained by the US government. We identified 93 MCs from these databases that were not included in any of the other sources (Excel Table S1). CCRIS has been discontinued, and the Carcinogenic Potency Database[64] has been adopted by a private company, Lhasa Limited. In addition, ToxValDB[62] and ToxRefDB[60] each include partially overlapping, incomplete subsets of bioassays conducted on pesticides. Some pesticides missing from ToxValDB and ToxRefDB include the food crop pesticides napropamide, acifluorfen, kinetin, and pyridate,[60,62] and the only way to access these and other pesticide carcinogenicity studies is through Freedom of Information Act requests, which are time consuming and inefficient, taking months or even years to receive documents in our experience. Furthermore, hundreds of chemicals tested in NTP bioassays were missing from ToxRefDB (314 chemicals) and ToxValDB (456 chemicals), and 629 chemicals were listed in either ToxRefDB or ToxValDB but not the other (Excel Table S5). Based on these inconsistencies, we anticipate that our list of 850 putative non-MCs is likely incomplete. Other sources we used to identify MCs (e.g., IARC,[54] *15th ROC*[56]) were not useful for identifying putative non-MCs because they are released infrequently and summarize data from many types of studies, including those where the mammary gland was not assessed. These limitations complicate attempts (such as this study) to identify mechanistic, structural, and other features that could be used to predict chemical carcinogenicity, delaying a shift away from time-consuming and expensive rodent studies.

In vitro *and mechanistic data.* Because many MCs were genotoxic, endocrine-active, or both (Excel Table S1), *in vitro* testing for these KCs can provide an HT approach to predict carcinogenicity and prioritize chemicals for *in vivo* testing, reducing reliance on animal models.[42] We relied on *in vitro* screens for ER activity and E2 and P4 steroidogenesis and on databases cataloging thousands of *in vitro* and *in vivo* genotoxicity assays. Although *in vitro* testing can efficiently identify potential hazards, as we have done here, there are limitations.

E2 and P4 steroidogenesis in H295R. There are several limitations with using the H295R steroidogenesis assay to identify chemicals that increase E2 or P4 levels in the breast. A biological limitation is that the assay measures steroid synthesis in an adrenocortical carcinoma cell line that expresses the full complement of metabolic enzymes involved in E2 and P4 synthesis from cholesterol.[177] Although these may predict systemic hormone changes, many cell types (including in the mammary gland,[178–181] ovary,[182,183] adipose tissue,[178,179] and skin[178]) are important sources of E2 and P4 production, affecting local hormone levels in tissues. In particular, estrogen levels in the breast are modulated by local aromatization of androgens into estrogens by preadipocytes.[180,181] As a result, hormone production in adrenocortical cells may not reflect the levels of hormones that would be produced in breast tissue. Comparison of H295R results with studies of hormone levels and tissue responses in the rat mammary gland would help in understanding the relevance to BC in humans.

A technical limitation of the H295R assay is that it is relatively insensitive to detecting E2 steroidogenesis, potentially leading to false negative results. Indeed, we previously noted that fold-increase of P4 synthesis tended to be more robust than that of E2 in this assay.[52] One possible explanation is that prestimulation with forskolin, which strongly increases E2 production, may reduce the assay's ability to measure induction of E2 by subsequent exposure to test chemicals.[71] In addition, we used Cardona and Rudel's classifications of effect size in H295R-CR, which

categorized chemicals that only increased hormone levels at the highest dose as borderline active, regardless of fold-change at that dose.[52] These high-dose effects, observed for 33 borderline E2 steroidogens and 26 borderline P4 steroidogens (see Cardona and Rudel supplemental Tables S1 and S2), may be relevant and important for human risk.

ER activation. A major strength of the ER activity data we used is that results from 18 independent *in vitro* assays were integrated with a computational network model,[73] minimizing potential false positives or false negatives from any individual assay. Notably, Judson et al. classified chemicals with an $AUC_{agonist}$ score ≥ 0.1 as ER agonists,[73] and we have listed chemicals with $AUC_{agonist}$ scores between 0.01 and 0.1 as borderline agonists as well, so some of these could be false positives.

Other considerations for identifying endocrine disruptors. The majority (196 of 278, Table 1) of chemical MCs had not been tested in the endocrine assays we relied on despite the fact that the US EPA has tested 2,012 chemicals in the H295R[71,72] and 1,812 in the ER pathway models. In all, only 71 MCs had been tested in both H295R and integrated ER activation analyses, and only 373 of the 920 BC-relevant chemicals had data for steroidogenesis, ER agonism, and genotoxicity. Although some of the MCs not tested may be incompatible with HT *in vitro* analyses owing to issues with chemical stability, volatility, or solubility, it is surprising that so many chemicals that induce rodent mammary tumors were not included in H295R or ER activity testing. The importance of expanding testing for endocrine effects is underscored by a study applying a set of chemical structure-based ER activity models to over 32,000 chemicals, where 4,001 chemicals were classified as "high priority actives" and 6,742 as "potential actives."[184] Application of the integrated *in vitro* testing and modeling approach developed by Judson et al.[73] to these predicted ER agonists would help identify additional ER-active chemicals beyond the 267 listed here. Similarly, we developed a quantitative structure-activity relationship (QSAR) model to predict chemicals that likely increase E2 or P4 steroidogenesis,[185] and *in vitro* testing of these chemicals could highlight additional chemicals that likely increase BC risk. Finally, in addition to chemicals not being tested for steroidogenic and ER activities, there are other endocrine pathways relevant to breast carcinogenesis (e.g., PR activation, prolactin signaling) that do not have reliable HT assays, so we were not able to include chemicals with these BC-relevant effects in this list.

It is also important to note that we assigned top EDC scores based on the ranking of chemicals included in the H295R-CR and ER activity screens, and these two assays are not directly comparable. For example, agonist AUCs calculated in Judson et al.[73] are normalized to 17-α-ethinylestradiol activity at the ER, whereas E2 and P4 steroidogens were classified by ranking potency and efficacy among chemicals with positive hit calls.[52] Sources of variability differ among the H295R assay, the 18 ER activity assays, and the computational integration of ER activity, so top EDC scores are a semi-quantitative approach to categorize two different types of effect sizes. In addition, incomplete endocrine activity screening for many chemicals can lead to underestimating EDC activity (e.g., P4 activity at the PR, steroidogens not tested for steroidogenesis), affecting statistical comparisons of top EDC scores among MCs vs. non-MCs.

Genotoxicity. We classified any chemical with a positive result in any relevant assay as genotoxic so as to capture many different types of genotoxicity. This approach does not distinguish potent genotoxicants from chemicals that are only active at very high concentrations. Although we recognize that a higher proportion of positive results provides greater confidence for a chemical's genotoxicity, different assays measure different aspects of genotoxicity, so a single positive result in a valid assay can reflect true genotoxicity and should not be negated by negative results in another assay. In addition, some chemicals may have been tested in formats that were not suitable to measure their genotoxicity (e.g., with or without metabolic activation, or at insufficient or excessive concentrations). It is also possible that chemicals we classified as nongenotoxic here were not tested in assays sensitive to their mode of genotoxicity. As a result, we may have included some false or misleading positives and negatives in our genotoxicity results.

A potential limitation of genotoxicity databases for predicting DNA damage in the breast is that activation of the ER can cause DNA damage in ER-responsive regions,[186–188] and observations of rearrangements in ER-responsive loci in breast tumors support that this is a clinically relevant process.[188] A recent study showed that the ER agonist propylparaben induces DNA damage in ER-expressing cells and rodent mammary glands, but not in ER-negative cells.[186] However, none of the databases used here classified propylparaben as genotoxic.[65,74–78] Given that typical genotoxicity testing is not performed in hormone-responsive cells,[189,190] the genotoxic effects of ER agonists may be missed. Of the references we used, only LCDB contained any record of cancer studies for parabens (butyl and isobutyl), which were negative under the conditions of the assay.[63] However, because exposure to parabens can elicit at least four Hallmarks of Cancer in mammary cells at environmentally relevant doses,[191] parabens are strong candidates for testing in a cancer bioassay that includes WoS.

Although many effective genotoxicity assays have been developed and performed on thousands of chemicals, chemical induction of genomic instability (a closely related KC) has proven more difficult to measure. Genomic instability refers to the continual and progressive cycle of DNA damage and mutagenesis (considered an "enabling characteristic" in the Hallmarks of Cancer[40]), and because prevailing theories suggest that cells require multiple mutations to become cancerous,[192–194] chemicals that induce genomic instability could be particularly relevant to carcinogenesis. Unfortunately, genomic instability is challenging to assess in HT because it requires multiple measurements over time. Developing methods to screen chemicals for their ability to induce genomic instability could address this gap.

Other considerations for *in vitro* and mechanistic data. A major limitation of *in vitro* assays is that most cannot replicate the complex biological processes that impact chemical toxicity. For instance, metabolism can render some compounds biologically active while other compounds are metabolized to nontoxic forms, and these processes also affect levels of chemicals and their metabolites in tissues.[195] Furthermore, most *in vitro* models contain only one cell type grown on plastic, whereas tissues contain many cell types that interact with each other and with surrounding stroma, so effects seen *in vitro* may be quite different from the actual effects of a chemical on tissue. Integration of toxicokinetics and toxicodynamics into *in vitro* assays and computational models will improve the ability for chemical screening to better reflect these complex mechanisms.

Finally, just as this list likely misses BC-relevant compounds that work by pathways that lack assays, the list also captures some compounds that are unlikely to increase BC risk in humans. In some cases, additional mechanistic information about chemicals on this list reveals activities that counteract their BC-relevant effects: for example, mifepristone increased P4 in H295R, but its key mechanism as a PR antagonist[196,197] likely mitigates the consequences of increased P4. Similarly, although aspirin increased E2 synthesis in the single-dose H295R assay, epidemiological studies have shown that aspirin reduces BC risk (reviewed by

Moysich et al.[198]) and improves survival,[199] possibly by reducing inflammation (another KC of carcinogens).

Implications of knowledge gaps for BC etiology. Although our list considerably expands the set of chemicals previously identified as BC-relevant by including ER agonists and E2/P4 steroidogens, it still likely misses many chemicals with BC-relevant activity because many biological processes relevant to breast carcinogenesis remain unknown. For example, the pesticide MC 3-iodo-2-propynyl-*N*-butylcarbamate was not steroidogenic, ER agonistic, or genotoxic, but further investigation could elucidate the pathways by which it causes mammary tumors and, in so doing, reveal mechanisms of toxicity that should be incorporated into chemical screening. Some BC-relevant chemicals that did not meet the criteria for inclusion in this study can also be identified through epidemiological studies, such as heavy metals and pentabromodiphenyl ethers,[83] and these could also be evaluated for KCs that increase BC risk.

In addition, some processes known to influence breast carcinogenesis do not have corresponding assays, lack publicly available chemical testing data, or have relevant assays that have only been performed on limited sets of chemicals. Mechanisms that we recommend prioritizing for assay development (especially in HT) and adoption into chemical screening programs include integrated models of activation of the PR, human epidermal growth factor receptor 2 (HER2), and epidermal growth factor receptor (EGFR) (analogous to the ER activity model used here); alterations in hormone metabolism; induction of inflammation; induction of genomic instability; BC-related gene expression and epigenetic signatures; and mechanisms of metastasis.[5,16,51,105,200–202] Assays for some of these mechanisms have been developed and are compatible with HT, such as the BCScreen[105] and ER modulator[203] gene expression panels, but they have not yet been adopted by chemical screening programs. We considered but did not use US EPA data from the PR_BLA screen for PR activity or from the NovaScreen aromatase activation assay because we found that results did not show reasonable signal-to-noise levels, reproducibility, or consistency with expected findings based on previous knowledge. Further development, validation, and application of these assays, and others targeting the mechanisms above, would significantly help integrate endocrine disruption and other understudied KCs into toxicology risk assessment.[136,204]

Mammary gland development is another BC-relevant endpoint that is incompletely understood and rarely assessed in toxicological studies. Interestingly, mammary developmental toxicants can increase susceptibility to mammary tumors whether they accelerate or delay gland development. For example, diethylstilbestrol, genistein, and bisphenol A (BPA) are ER agonists that accelerate mammary gland development,[94,96,205,206] and each has been shown to induce mammary tumors[7,56,63,65,207,208] (although we did not list BPA as an MC because our source databases did not include the relevant studies). On the other hand, atrazine[99,209] and TCDD[96,100] delay mammary gland development, and atrazine is an MC that increases E2 and P4, and TCDD sensitizes the mammary gland to DMBA-induced tumors,[210] but it was not included in steroidogenesis or ER activity screening (Excel Table S4). Given the significant overlap between MCs, EDCs, and mammary development-disrupting chemicals, other mammary development disruptors are also likely to be EDCs and possibly MCs. Further investigation into the hormonal and tumorigenic effects of mammary development disruptors could provide insight into other mechanisms of BC development.

Translation and Implications

Our list of BC-relevant chemicals and their KCs can immediately guide regulatory prioritization, product formulation, and consumer disclosures, while also setting the stage for future research. This approach is useful for flagging chemicals with activity relevant to common human diseases, and we hope others will employ it to similarly prioritize chemicals for preventive action based on their biological activities and potential to affect human health.

Our analysis highlights actions that can be taken by regulatory and testing agencies, such as the US EPA and NTP, to identify and reduce risks posed by potential breast carcinogens. We identified hundreds of MCs and other BC-relevant chemicals that lack adequate data for genotoxicity and endocrine disruption, and these could be prioritized for *in vitro* and *in vivo* toxicity testing. Based on their mechanisms of concern, we argue that many of the chemicals on this list should not be considered low hazard without rigorous evaluation of their potential to adversely affect the breast. Similarly, because of limitations that we have discussed above in mammary tumor assessments in rodent cancer bioassays, we suggest prioritizing the genotoxic EDCs on our list for testing in a cancer bioassay that is sensitive to mammary effects and captures important WoS, unless such studies already exist. We recommend that regulatory guidelines for *in vivo* carcinogenicity testing be updated to standardize approaches for mammary gland analysis and interpretation,[44,52,58,83,136,138,211] and previous studies where mammary effects were discounted should be reevaluated. We consider the 56 putative non-MCs that are genotoxic EDC+ to be priority candidates for such a review.

Another priority is to develop additional *in vitro* and short-term assays that extend our ability to capture relevant KCs for breast carcinogens. As described above, many KCs of carcinogens lack efficient screening methods, impeding efforts to use this framework to identify cancer risk factors.

In addition to filling data gaps in chemical screening, identifying BC-relevant chemicals and their KCs can support a range of future inquiries. For example, this study could be used to develop QSAR models for flagging structural features common to chemicals with different combinations of genotoxic, steroidogenic, and ER-agonistic activities, such as the one we have recently published for E2/P4 steroidogens,[185] supporting chemical read-across and predictive toxicology. Similarly, a study of genotoxic EDCs that did not induce mammary tumors in a bioassay could provide insights about chemical features that prevent the anticipated mammary tumors from developing.

The BC-relevant chemicals and mechanisms identified here can also guide biomonitoring and epidemiological studies. Monitoring exposures to these BC-relevant chemicals in humans, particularly those with predicted high exposure, could identify high-risk demographics, geographic regions, or important sources of exposure. Prioritizing chemicals that people are exposed to chronically can expedite preventive action. Epidemiological studies can incorporate these exposures, considering the potential impact of coexposures and using quantitative potency and efficacy data from *in vitro* assays to develop evidence-based exposure metrics.[124] Epidemiological studies that consider exposure patterns of BC-relevant chemicals could also shed light on why BC rates have surpassed lung cancer rates in the United States[2,3] and worldwide.[1] We are preparing to publish a companion manuscript that summarizes predicted exposure sources and intake levels of these BC-relevant chemicals in the United States, and this can provide additional direction for biomonitoring, epidemiology, and risk reduction.

Conclusions

This list of 921 BC-relevant exposures, 279 of which induce mammary tumors *in vivo*, provides an updated and more comprehensive understanding of chemical exposures that may increase BC risk. By classifying chemicals according to their ability to induce synthesis of E2 or P4, activate ER signaling, and create

DNA damage and mutations, this list establishes a new basis for chemical hazard assessment and risk reduction. We demonstrated that MCs were significantly enriched for these mechanisms compared with both putative non-MCs and with all chemicals tested, especially for stronger endocrine effects. Integrating evidence for these two KCs could help predict whether a chemical is likely to be an MC and, by inference, increase BC risk. Interestingly, MCs were more significantly enriched for increasing both E2 and P4 synthesis than either hormone alone, and they were more likely to be steroidogens than ER agonists, indicating that steroidogenesis warrants more emphasis in future studies of chemicals that increase BC risk.

This list can inform biomonitoring and epidemiological studies, strengthen testing of chemicals for carcinogenic properties, support application of the KC approach for predicting carcinogens, and prioritize chemicals for revised risk assessments or testing in a cancer bioassay with BC-relevant WoS. Based on their activity in two BC-relevant KCs, we argue that many of these chemicals should not be considered safer alternatives or low hazard without additional investigation of their ability to impact the breast. Future structural analyses of these chemicals can also provide a basis for read-across methods to identify other potential BC-relevant chemicals or chemical classes. In addition, this study models a process for identifying, and integrating into toxicological testing, key biological processes common to chronic diseases. Together, we provide a springboard for a wide range of actions that could improve our understanding of BC etiology and our ability to prevent the leading cause of cancer death among women worldwide.

Acknowledgments

We are grateful to Abigail Bline and Alexandre Borrel for checking code written by J.E.K. We thank Richard Judson for providing downloadable versions of the ToxValDB and eChemPortal genotoxicity databases, Lhasa Limited for providing a table of chemicals with positive or equivocal evidence for mammary tumors, Katie Paul-Friedman for assistance in processing data from ToxRefDB, and Claudia Polsky and Suzanne Fenton for reviewing and providing feedback on the manuscript. This work was supported by charitable donations to the Safer Chemicals Program at Silent Spring Institute and by the California Breast Cancer Research Program grant 23QB-1881.

Silent Spring Institute is a scientific research organization dedicated to studying environmental factors in women's health. The institute is a 501(c)3 public charity funded by federal grants and contracts, foundation grants, and private donations, including from BC organizations. Study funders had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

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